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Development of a gene deleted pseudorabies (Aujeszky's disease) vaccine and evaluation of a differential diagnostic serology test

Donald Raymond Cook
Iowa State University

Development of a gene deleted pseudorabies (Aujeszky's disease) vaccine and evaluation of a differential diagnostic serology test

by

Donald Raymond Cook

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EXPLANATION OF THESIS FORMAT

The following thesis consists of a general introduction, a review of literature, two separate manuscripts (Sections I and II), and literature cited. The master's candidate, Donald Raymond Cook, is the senior author and principal investigator for each of the manuscripts.

GENERAL INTRODUCTION

Pseudorabies is an infectious disease caused by pseudorabies virus (PRV), which is classified into the herpesvirus subfamily, Alphaherpesvirinae.[72] Herpesviruses have relatively large genetic potentials (50 to 100 genes)[99] and complex life cycles, involving infection of multiple cell types and the establishment of latent infection within the host. Some PRV genes are not essential for replication and production of virus particles, particularly when the virus is propagated in cell cultures. It is possible for genetic mutation to create attenuated strains of virus, and this has occurred with high passages of virulent PRV in cell cultures[81] and chicken embryos.[56] A number of these attenuated strains of PRV have been evaluated as vaccines.[8,9,18,63,90,95] They have been selected mainly on the basis of reduced virulence, without knowing the nature or stability of the underlying genetic alteration.[59] Although vaccination based on these attenuated strains has been effective, there are some disadvantages to their use.[73] Planned modifications to the PRV genome, using recombinant DNA technology, have been made to produce vaccine strains that overcome these disadvantages. The goal has been to produce vaccine strains that provide high levels of protection against disease, that are safe for all classes and ages of pigs, that have reduced virulence for other species, and that allow serological differentiation between vaccinated and field infected pigs.[94]

LITERATURE REVIEW

Introduction

The first scientific description of pseudorabies was by Aujeszky in 1902,[6] however, there is evidence to suggest that PRV has been present in the United States since the mid 1800s.[43] Pigs are considered the reservoir host of PRV[22,46] and the only significant source of infection of other susceptible species. Pseudorabies has a wide host range including cattle, sheep, goats, dogs, cats, and raccoons, and in these species the disease is uniformly fatal.[28,38,53] The disease in pigs is economically important, and characterized by abortions, neurological signs in piglets, and respiratory signs and growth retardation in growing pigs.[105] Mortality due to PRV infection is greatest in baby pigs and least in mature pigs. The severity of infection is dependent on the virulence of the viral strain, age of the pigs, dose of virus and route of exposure.[28] Prior to the 1970s there were only sporadic reports of outbreaks of pseudorabies in pigs in the United States,[80,87,89] however, in the first half of that decade the incidence of pseudorabies significantly increased.[26,37] Slaughter survey serums collected in the United States in 1974 showed 0.56% of pigs were positive for PRV.[29] By 1978 the number of positives had increased to 3.73%.[96] As pseudorabies was considered a serious threat to the pig industry, considerable attention was given to measures that would reduce losses to the disease.[39] Attenuated and killed PRV vaccines were licensed for use in the United States in 1977 and 1978 respectively,[12,55] and federal regulations prohibiting the interstate movement of seropositive pigs were put into effect by 1979.[12]

Vaccination of pigs with killed or modified live virus is now commonly practiced, as it will reduce economic losses due to pseudorabies.[24] The vaccines licensed in the United States are shown in Table 1. Vaccination may help eliminate PRV from a pig herd by decreasing the shedding of virulent virus after challenge and reducing the spread of virus within a herd.[22] However, it is not as effective in controlling pseudorabies as the elimination of infected pigs from the herd by test and removal procedures,[97] so these methods are often used in combination. Vaccines reduce or prevent clinical signs in pigs exposed to PRV, but they do not prevent infection or the establishment of latent infections.[70] Therefore, there is a need to assess PRV exposure in vaccinated pigs. The standard serological procedures used to detect PRV antibodies are of little value for this purpose. They cannot distinguish antibodies induced by natural infection with PRV from antibodies induced by PRV vaccines.[48] Considerable effort has been directed at this problem, as the inability to differentiate these antibody responses markedly decreases the efficiency of eradication programs utilizing vaccination.[101] The development of gene deleted vaccines and their associated diagnostic tests, specific for antibodies to the protein coded for by the deleted gene, makes the distinction possible.[107] Currently, the only gene deleted vaccine which has an accompanying USDA licensed diagnostic test is PRV/Marker®[a]. This vaccine utilizes a genetically engineered PRV strain in which the deletion of the gene coding for glycoprotein X acts as a "negative marker", allowing serological differentiation between

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[a] SyntroVet Incorporated, Lenexa, Kansas.

Table 1. Licensed Pseudorabies Vaccines at 1/1/89

Vaccine	Company	Accompanying Diagnostic Test	Modified Live (ML)/Killed (K)
PRV/Marker®	SyntroVet Inc.	Yes	ML
PR-Vac®	Norden Labs.	No	ML
PR-Vac®-Killed	Norden Labs.	No	K
PR-Vac®/Leptoferm-5®	Norden Labs.	No	ML
Pseudorabies Vaccine	Bio-Ceutic Labs.	No	ML
Omnivac™-PRV	TechAmerica	No	ML
Pseudovax	Pitman-Moore	No	ML
Tolvid®	The Upjohn Co.	No	ML
Suvaxyn™-PRV	Solvay Veterinary	No	K
Porci-Rab®	Beecham Labs.	No	K

vaccinated pigs and those infected with field virus.[94]

In the following review of the literature on PRV, emphasis will be placed on the physio-chemical properties of the virus, the genetic variation between vaccine strains of PRV, vaccination of pigs against pseudorabies, and the humoral immune response in pigs induced by vaccine and natural exposure to PRV.

Pseudorabies Virus

The classification of PRV (Suid herpesvirus I) as a member of the family Herpesviridae is based on its physicochemical properties and strategy of replication.[16,35,72] The PRV genome is a double stranded, linear DNA molecule with a molecular weight of 90 X 10^6 daltons.[16] The nucleocapsid has a diameter of 150 to 186 nanometers as measured by electron microscopy.[16] This is surrounded by an envelope consisting of a double or triple lipid containing membrane.[23] Pseudorabies virus has further been classified into the subfamily Alphaherpesvirinae based on its variable host range, highly cytopathic nature, short replication cycle and frequent establishment of latent infection.[72]

The genome of PRV consists of approximately 160 kbp,[85] although this may vary due to major differences between wild and some vaccine strains of the virus.[58] In recent years, there have been rapid advancements in the knowledge of the genomic structure of PRV. These advancements have come mainly from the study of mutant strains of PRV by using restriction endonucleases.[16,17,69,76,77,79,108] There are 4 distinct regions of the genome: a unique long (UL) region of 65 X 10^6 daltons, an inverted repeat (IR) region and

terminal repeat (TR) region of 10 X 10^6 daltons each, and a unique short region of 6 X 10^6 daltons.[16,17]

The number of polypeptides identified as being coded for by the PRV genome, has changed over time with advances in protein separation and resolution techniques.[16] Of the 16 structural proteins identified, the virus envelope contains 4 major and 3 minor glycoproteins and 1 non-glycosylated protein.[42,60] The other 8 are non-glycosylated proteins found in the nucleocapsid.[93] Mapping of PRV genomes has identified the locations of some of the genes that code for these proteins and also identified major genetic differences between strains.

The unique short region of the PRV genome has had significant attention from researchers, as most avirulent strains used as vaccines, have genetic deletions in this region. The unique short region contains a cluster of 4 glycoprotein genes, glycoprotein X (gpX),[81] glycoprotein 50 (gp 50),[108] glycoprotein 63 (gp 63),[77] and glycoprotein I (gp I).[69]

The PRV strains used in PR-Vac®[b] and Pseudorabies Vaccine[c] do not code for gpI.[73] Glycoprotein I, is one of the minor envelope glycoproteins[56] and plays a role in the virulence of PRV by being involved in the release of virions from some cell types.[14]

Both genetically engineered strains of PRV used as vaccines in the United States, PRV/Marker® and Tolvid®[d], have deletions of the gene coding for gpX.[73,94,98] There is controversy whether or not gpX is a non-structural

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[b] Norden Laboratories, Lincoln, Nebraska.
[c] Bio Ceutic Laboratories, St. Joseph, Missouri.
[d] The Upjohn Company, Kalamazoo, Michigan.

glycoprotein. It is secreted from PRV infected cells and early workers reported that it did not form part of the enveloped virus.[15] However, recent evaluation of the gpX gene sequence suggests that the primary translation product is probably a membrane protein with the usual hydrophilic cytoplasmic domain structure.[13] There is a cellular form of gpX with a higher molecular weight than the form released from infected cells.[13] There are no reports of naturally occurring PRV strains which lack the gpX gene, however, it is not essential for growth in cell cultures or replication within the host.[99] As yet, the function of gpX has not been determined.

The thymidine kinase (TK) gene, located in the UL region of the genome, codes for an enzyme that provides the virus with a thymidine utilization pathway independent of the cell thymidilate synthetase pathway.[86] This TK activity is not essential for growth of PRV in cell cultures;[95] but appears important for virulence and latency.[99] The TK gene has been deleted from genetically engineered stains of PRV to reduce vaccine virulence and to lower the potential of vaccine strains to establish latent infections.[73]

The PRV strain used in PRV/Marker® has additional alterations to its genome. Two small gene sequences have been deleted from the IR and TR regions to reduce virulence and a lactase gene has been inserted to allow rapid identification of isolates of this strain.[37]

Pseudorabies Virus Vaccines

The evaluation of a number of modified live pseudorabies virus (MLPRV) vaccines and killed pseudorabies virus (KPRV) vaccines have been

reported in the literature. There is considerable variation in the reported findings due mainly to difference in biological characteristics between the strains of PRV used in MLPRV vaccines, but also due to different methods employed for inactivation of the KPRV vaccines.

Vaccination with MLPRV or KPRV will not prevent infection[3,25,27,63,70] or the establishment of latent infection with virulent PRV.[70] Vaccination will prevent mortalities in piglets and reduce clinical signs, including fever, weight loss and central nervous system (CNS) dysfunction, after challenge exposure.[3,25,27,70] It will also reduce virus dissemination after challenge exposure, but not prevent it.[3,11,25,27,63] It is likely that a more limited range of organs are infected by virus in vaccinated animals and there is a reduction in virus replication in those organs that are infected.[27]

Modified Live Vaccines

Modified live virus vaccines generally require virus replication within the host animal in order to produce sufficient antigenic mass to stimulate the immune system.[73] The replication and distribution of PRV in pigs, immunized intramuscularly (IM), is dependent on the strain and amount of virus used.[11,27,63] The advantage of replication within the host is that generally a single dose of MLPRV is sufficient to produce a good immune response and proper immunization.[3,18,25,27,63,90] Compared to KPRV vaccines, MLPRV vaccines have an advantage of inducing an immune response that may mimic that produced by natural infection in the pig.[73] Vaccination with MLPRV is the method of choice for controlling clinically active pseudorabies outbreaks, as it will rapidly reduce clinical losses.[73]

There are some potential disadvantages in using modified live vaccines. First, reversion to greater virulence during replication in the host animal is a potential problem,[76] although reversion of MLPRV vaccines has not proved a problem in practice.[25,73] Second, the excretion of modified live virus is another potential hazard,[63,73] because it may initiate a series of passages through pigs, possibly leading to an increase in virulence of the vaccine virus.[25] Excretion of MLPRV from pigs vaccinated IM has been reported[25] with strains MK-25[95] and B-KAL 68,[90] although a number of researchers have found no evidence of excretion using other strains.[4,9,25,50,63] Third, unrecognized adventitious or contaminating agents may infect the cells in which the vaccine is produced. Adventitious viruses including avian leukosis and other retroviruses, SV-40, bovine viral diarrhea virus, and porcine parvovirus have been found in various modified live vaccines.[73] Fourth, as clinical signs may be seen in pigs following vaccination with MLPRV[25] there may be some risk in administering these vaccines, particularly to young or pregnant animals. Tolvid® is the only USDA licensed vaccine not recommended for pigs less than 3 weeks of age.[73,98] There are no reports of reproductive failure in pregnant animals due to MLPRV vaccination. Modified live PRV vaccines have varying degrees of safety in sheep, cattle, cats, dogs and laboratory animals.[73,100] Fifth, there is the potential for MLPRV to recombine with other virus strains. The recombination of vaccine virus, containing gene deletions, with other viruses is unlikely. However, if it occurred it may give misleading results on serological diagnostic tests that differentiates vaccinated from field infected pigs.[73]

Killed Virus Vaccines

Killed virus vaccines, prepared by inactivating whole virions, generally stimulate the development of circulating antibody against viral coat proteins.[67] A number of methods have been reported for the chemical inactivation of PRV so it can be used as a killed vaccine.[44,49,75,102,112,113] Proper inactivation overcomes the disadvantages associated with MLPRV vaccines that stem from replication of virus within the host or from contamination of vaccines with adventitious agents.[67,73]

A major disadvantage of killed virus vaccines has been that the immunity conferred by one dose is often low and of short duration, and a second injection must be administered. The use of two doses of KPRV vaccine is recommended for adequate protection against virulent PRV in the field.[74] Most reports indicate that KPRV vaccines are less effective than MLPRV vaccines[25,36,63,64] although identical efficacy has been reported.[3,25]

Although there is incomplete knowledge of the factors controlling the class and intensity of an immune response, it is influenced by the molecular and chemical complexity, dose, and form of administration of the inducing agent, and the immunogenic capacity of the responding host.[103] The dose of inducing agent and the form in which it is administered are two easily controllable variables that can be manipulated to improve the immunogenicity of killed vaccines.[73] The dose of virus used in killed vaccines is a compromise between the level of immunity required and the cost of vaccine production. The dose can be lowered by effective use of adjuvants and carriers in which the antigen and adjuvants are incorporated.[52] Adjuvants act nonspecifically to increase specific

immune response to an antigen by inducing the production of lymphocytic growth factors.[2,51] There are potential undesirable effects of adjuvants including; unacceptable inflammatory or necrotizing reactions at injection sites, CNS effects, impairment of growth, arthritis, induction of autoimmune responses, and the rendering of food animals unsafe for human consumption.[1,2,20] Only adjuvants that are both efficacious and lacking in side effects can be used in killed virus vaccines.

Humoral Immune Response

The humoral immune response of pigs to PRV infection and vaccination have been studied extensively using a number of techniques which detect circulating antibodies. Some of these tests include: microtitration serum neutralization test (SVN),[45] microimmunodiffusion test,[40] macroimmunodiffusion test,[91] indirect solid-phase microradioimmunoassay test,[54] indirect immunoperoxidase method,[84] radioimmunoprecipitation method (RIP),[65,108] complement fixation test,[33] latex macroagglutination test,[109] and enzyme-linked immunosorbent assays (ELISA).[19,31,35,48,83,92,107]

In PRV infected animals, anti-viral antibodies can be detected by ELISA at 5 days post-challenge (PC).[83] Up to day 7 PC, these antibodies are almost exclusively of the IgM class. IgM titers are still high at day 18 PC but then rapidly decrease.[61] Antibodies of the IgG class are detectable at day 7 PC,[83] peak at about day 35 PC and remain detectable by ELISA and SVN for an extended period.[41,61,62,78,83] Low levels of IgA class antibodies are secreted by oral and nasal mucosa shortly after PRV infection.[10,83] Re-exposure to PRV

induces high levels of protective anti-PRV IgA and also a marked circulating SVN antibody response.[10,11]

Vaccination of pigs with PRV vaccines induces a humoral immune response that differs from that observed in natural infection. It has been demonstrated using an ELISA that in PRV vaccinated pigs, IgM antibodies decline more rapidly, IgG antibodies peak at lower titers, and IgA antibodies cannot be detected in oropharyngeal fluids.[83] The SVN antibody titers of vaccinates are generally lower than those of PRV infected pigs, and there is marked variability in the persistence of these antibodies with different PRV vaccines.[25,27,61,63,64] In some studies, pigs which received 1 dose of a KPRV vaccine had higher SVN antibody titers of longer duration than pigs which received MLPRV vaccine.[64,114] Exposure of vaccinated pigs to PRV produces a marked anamnestic SVN antibody response.[3,25]

The immunogens of the pseudorabies virion have not been fully characterized, however, it is known that glycoproteins induced by PRV infection and those ultimately included in the mature enveloped virus are immunologically important. There may be significantly lower humoral immune response in animals exposed to PRV strains lacking genes coding for some glycoproteins. Those glycoproteins that have been identified as immunogens include: gpI, glycoprotein II, glycoprotein III, gpX, gp50, and gp63.[32]

Using a gpI ELISA, it has been shown that antibodies to gpI persist in pigs for at least 32 weeks PC with PRV, and the antibodies can also be detected in pigs vaccinated with PRV strains that contain the gene coding for gpI.[106] In contrast, gpI antibodies have not been reported in pigs vaccinated with PRV strains in which there is deletion of the gene coding for gpI (gpI negative).[104]

The use of gpI negative vaccines in combination with culling of gpI antibody seropositive infected pigs has achieved elimination of field virus from PRV infected herds.[104]

Pigs infected with field strains of PRV produce antibodies to gpX detectable by HerdChek®: Anti-PRV-gpX[f] assay.[48] The subunit diagnostic antigen (SUDA) ELISA and RIP detect antibodies to SUDA,which has a reported molecular weight the same as that as gpX.[65] McGinley and Platt detected SUDA antibody as early as 14 days after low dose PRV challenge of PRV subunit vaccinated pigs. Antibody persisted as long as 113 days PC in 3 of 10 pigs, but 1 pig became seronegative by SUDA ELISA as soon as 21 days PC. Eight of these 10 pigs were shown to be latently infected, and 1 latently infected pig failed to produce SUDA antibodies after viral recrudescence. Antibodies to gpX have no PRV neutralizing activity and pigs with gpX antibodies alone, are not protected from lethal PRV infection.[99] Vaccination of pigs with PRV strains, in which there is a deletion of the gene coding for gpX (gpX negative), does not induce gpX antibody production.[48]

Glycoprotein III and gp50, induce the formation of antibodies which have a neutralizing activity in the absence of complement.[42,108] Virus neutralizing antibody is primarily responsible for neutralizing free virus in the blood and tissue fluids.[10] The presence of neutralizing antibody, does not prevent multiplication or distribution of PRV in tissues of vaccinated pigs challenged with PRV, and the range of tissues in which these occur is the same as

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[f] IDEXX, Incorporated., Portland, Maine.

challenged pigs. However, it has been demonstrated that virus titers are lower in the first 7 days PC in vaccinated animals.[111]

Despite attempts by a number of researchers,[21,25,61,110] it has not been possible to correlate immunological parameters with the effectiveness of PRV vaccines in limiting the clinical sequelae of PRV infection. It would appear that the level of protection provided by vaccination is a function of both the humoral and cell-mediated immunity induced.[5,111]

SECTION I. DEVELOPMENT OF KILLED VACCINES CONTAINING A GPX DELETED STRAIN OF PSEUDORABIES VIRUS

SUMMARY

The efficacy of 13 inactivated vaccine preparations containing a glycoprotein X (gpX) gene deleted strain of pseudorabies virus (PRV) was evaluated by challenging vaccinated pigs intranasally. Experimental vaccines contained 1 of 4 adjuvants and varying concentrations of viral antigens. Vaccination of pigs with 1 dose of experimental vaccines adjuvanted with 50% Montanide ISA 50 or 20% Syntrogen induced a protective immunity at least equal to that induced by 2 commercially available killed PRV vaccines also evaluated. The serum virus neutralizing antibody titers induced by the experimental vaccines containing Montanide ISA 50 were much higher than those induced by the commercially avaiable vaccines. None of the experimental vaccines induced gpX antibodies, detectable by the HerdChek®: Anti-PRV-gpX assay, in vaccinated pigs. Therefore, this assay could differentiate PRV vaccine induced antibodies from antibodies induced by natural exposure when used in conjunction with these experimental vaccines.

INTRODUCTION

The vaccination of pigs against pseudorabies is commonly practiced.[9,17] Vaccination will not prevent infection with pseudorabies virus (PRV); however, it will reduce the duration and severity of clinical signs of pseudorabies and the amount of virus shed by infected pigs.[1,4,5,14] Modified live pseudorabies virus (MLPRV) vaccines are generally considered to provide greater protection than killed pseudorabies virus (KPRV) vaccines,[4,8,13,14] even though the antibody titers that are induced by one dose of KPRV vaccine are generally of similar magnitude and duration as titers induced by one dose of MLPRV vaccine.[4] Killed PRV vaccines do have some advantages over MLPRV vaccines. A higher anamnestic response is induced in sensitized animals with KPRV vaccines.[4,14,17,21] Also the inactivation of killed vaccines prevents vaccine virus from replicating in vaccinated animals. Replication of virus does occur in the pig after MLPRV vaccination.[3,5,13] There are potential problems that may result from vaccine virus replication. Vaccine virus may be excreted,[4] leading to a series of passages through pigs and possibly an increase in virus virulence by genetic mutation or recombination.[17] Replication of some MLPRV in pigs causes mild clinical signs,[4] although it is possible that in young or pregnant animals virus replication may produce more severe effects.[17] For these reasons, KPRV vaccine is considered by many veterinarians as the vaccine of choice for boostering immunity, particularly in pregnant sows.[17]

A major disadvantage in using KPRV vaccines is that none of the KPRV vaccines available in the United States have an accompanying diagnostic test that can differentiate antibodies induced by vaccination from antibodies

induced by field virus infection. In contrast, pigs vaccinated with the commercially available MLPRV vaccine, PRV/Marker®[a] can be distinguished from PRV infected pigs by using the HerdChek®: Anti-PRV-gpX assay[b] (Anti-gpX ELISA).[12] Specific modifications have been made to the PRV/Marker® vaccine virus, including the deletion of the gene coding for glycoprotein X (gpX).[20] The deletion of this gene acts as a "negative marker." The Anti-gpX ELISA is specific for antibodies to gpX and ignores antibody titers in pigs vaccinated with PRV/Marker®. However, it detects gpX antibody in pigs infected with field strains of PRV.[12] The development of a KPRV vaccine that does not induce gpX antibody in vaccinated pigs and that could be used in conjunction with the Anti-gpX ELISA would be beneficial in eradicating PRV from infected herds.

The purpose of this study was to evaluate inactivated vaccine preparations containing a gpX deleted strain of PRV. The virus strain was specially developed for this study and provided by SyntroVet.[a] The type of adjuvant and the concentration of viral antigen in vaccine preparations were varied to determine the effect these parameters had on the level of immunity induced in vaccinated pigs. The level of immunity was evaluated by measuring the humoral immune response to vaccination and by comparing the effect that intranasal challenge with PRV had on vaccinated and non-vaccinated pigs.

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[a] SyntroVet Incorporated, Lenexa, Kansas.
[b] IDEXX Incorporated, Portland, Maine.

MATERIALS AND METHODS

General

Vaccines

Thirteen experimental vaccine preparations and two commercially available vaccines were used.

Experimental vaccines were supplied by SyntroVet Inc. The SyntroVet virus strain was propagated on Vero cells to obtain the viral antigens contained in the experimental vaccines. Infected cultures were harvested 48 to 72 hours post-infection. The infectivity titer of viral fluids was determined by plaque assay[6] prior to the inactivation of virus with binary ethyleneimine.[2] After inactivation, viral fluids were mixed with either 1 of 4 adjuvants; Emulsigen[c], Amphigen Base[d], Montanide ISA 50[e], or Syntrogen™[a], according to the adjuvant manufacturers recommendations.[11,16,19] The final concentration of viral antigen in vaccine preparations was estimated from the pre-inactivation infectivity titers, and expressed as a 1X concentration (undiluted viral fluids) or the respective fraction of the 1X viral fluids.

The two commercially available KPRV vaccines used, PR-Vac®-Killed[f] and Porci-Rab®[g] were purchased anonymously.

All vaccines were administered as a 2 ml dose, intramuscularly (IM) in the neck.

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[c] Modern Veterinary Products Incorporated, Ralston, Nebraska.
[d] Hydronics Incorporated, Omaha, Nebraska.
[e] Seppic, Paris, France.
[f] Norden Laboratories, Lincoln, Nebraska.
[g] Beecham Laboratories, Bristol, Tennessee.

Animals and Housing

All pigs were obtained from pseudorabies free herds and housed in facilities secure for PRV exposure.

Challenge virus

Pseudorabies virus strain VDL 4892 was used to challenge vaccinated and non-vaccinated pigs. The virus was propagated and titrated on Madin Darby bovine kidney (MDBK) cells. Growth medium consisted of Minimal Essential Medium[h] (MEM) supplemented with 50 mg/ml gentamycin sulphate, 2 mg/ml amphotericin B and 10% fetal bovine serum. Maintenance medium was MEM supplement with 400 mg/ml streptomycin sulfate, 100 units/ml penicillin, 50 mg/ml gentamycin sulfate, 2 mg/ml amphotericin B, 24 units/ml tylocine and 2% fetal bovine serum.

Virus isolation

Nasal and tonsil swabs and tissue samples were assayed for virus. Nasal and tonsil swabs were placed in tubes containing 0.5 mls of Earles[h] medium. The tube was then vortexed, the swab removed and the tube centrifuged at 3000 PRM (2000 g.) for 10 minutes. Tissue samples were mascerated in an equal volume of Earles medium and centrifuged as described for swabs. Supernatants were assayed for PRV by inoculation in MDBK cells which were examined for CPE daily for 7 days. Negative cultures were subcultured and observed for 7 days. Pseudorabies isolates were identified by immunofluorescence.

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[h] Gibco Laboratories, Grand Island, New York.

Antibody assays

Serum samples were assayed by the serum virus neutralization (SVN) test as described by Hi11 et al.,[10] and the Anti-gpX ELISA using the recommended procedure.[12]

Trial 1

Vaccines

Three vaccines containing inactivated SyntroVet virus (KV-SV) were used: vaccine lA containing 0.004X viral antigen and 40% Emulsigen/dose (0.004X viral antigen/40% Emulsigen), vaccine lB - 0.012X viral antigen/40% Emulsigen, and vaccine lC - 0.04X viral antigen/40% Emulsigen.

Procedures

Twenty-five pigs, 4 to 5 weeks of age were randomly assigned to 5 groups of 5 pigs. Three groups were vaccinated twice at 21 day intervals with 1 of each of the 3 vaccines. A fourth group received 1 dose of vaccine lB at the time of the second vaccination of the other groups. One group remained non-vaccinated controls. Twenty-one days after the second vaccination, all pigs were challenged intranasally with $10^{3.4}$ PFU of PRV as a 1 ml dose. Clinical observations were made, nasal and tonsil swabs collected and rectal temperatures recorded daily for 14 days post-challenge (PC). Serums were obtained from blood samples collected on the day of vaccination and then weekly until 14 days PC. Pigs were weighed 14 days PC. Pigs that died during the trial were necropsied and samples were collected for virus isolation.

Trial 2

Vaccines

Three KV-SV vaccine preparations were used: vaccine 2A - 0.1X viral antigen/20% Emulsigen, vaccine 2B - 0.1X viral antigen/20% Emulsigen/67.5% Novalep®-5[i] , and vaccine 2C - 0.1X viral antigen/10% Amphigen Base. In addition, two commercially available vaccines, PR-Vac®-Killed and Porci-Rab® were used.

Procedures

Twenty-nine pigs, 4 to 5 weeks of age were randomly assigned to 5 groups of 5 pigs and 1 group of 4 pigs. Each of the groups containing 5 pigs were vaccinated with 1 dose of vaccine and the group of 4 pigs remained non-vaccinated controls. Forty-two days post-vaccination (PV) all pigs were challenged intranasally with $10^{5.8}$ PFU of PRV as a 1 ml dose. Serums were collected on the day of vaccination and then weekly until 14 days PC. Pigs were observed for clinicals signs daily for 14 days PC; weighed on the day of challenge and day 14 PC; and nasal swabs were collected on days 7, 10 and 14 PC. Pigs that died during the trial were necropsied and brain, lung, tonsil, and spleen samples were collected for virus isolation.

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[i] Coopers Animal Health Incorporated, Kansas City, Missouri.

Trial 3

Vaccines

Four KV-SV vaccine preparations were used: vaccine 3A - 1X viral antigen/30% Emulsigen, vaccine 3B - 1X viral antigen/10% Syntrogen, vaccine 3C - 0.2X viral antigen/10% Syntrogen, and vaccine 3D - 1X viral antigen/50% Montanide ISA 50.

Procedures

Twenty-five pigs, 3 to 4 weeks of age were randomly assigned to 5 groups of 5 pigs. Four groups were vaccinated with 1 dose of vaccine and the other group remained non-vaccinated controls. Twenty-six days PV pigs were challenged intranasally with $10^{4.8}$ PFU of PRV as a 1 ml dose. Serums were collected on the day of vaccination and days 7, 14 and 21 PV, and on the day of challenge (day 26 PV) and days 7 and 14 PC. Pigs were weighed on the day of vaccination, the day of challenge, and day 14 PC. Pigs were observed for clinical signs daily for 14 days PC, and nasal swabs were collected on days 3, 6, 9 and 12 PC. The vaccination site was palpated on days 7, 21, and 40 PV to detect any tissue reactions to the vaccines. Pigs that died during the trial and the pigs vaccinated with preparations containing Montanide ISA 50 were necropsied.

Trial 4

Vaccines

Four KV-SV vaccine preparations were used: vaccine 4A - 1X viral antigen/50% Montanide ISA 50, vaccine 4B - 0.5X viral antigen/50% Montanide

ISA 50, vaccine 4C - 0.2X viral antigen/50% Montanide ISA 50, and vaccine 4D - 0.5X viral antigen/20% Syntrogen.

Procedures

Twenty-five pigs, 3 to 4 weeks of age were randomly assigned to 5 groups of 5 pigs. Four groups were vaccinated with 1 dose of vaccine and the other group remained non-vaccinated controls. Twenty-two days PV pigs were challenged intranasally with $10^{4.8}$ PFU of PRV as a 1 ml dose. Serums were collected on the day of vaccination and days 7 and 14 PV, and on the day of challenge (day 22 PV) and days 7 and 14 PC. Pigs were weighed on the day of vaccination, the day of challenge, and day 14 PC. Pigs were observed for clinical signs daily for 14 days PC and nasal swabs were collected on days 3, 6, 9 and 12 PC. The vaccination site was palpated on days 7, 22 and 36 PV.

RESULTS

Trial 1

Response to vaccination

A single vaccination with any of the vaccines did not produce detectable SVN antibody to PRV within the 21 days prior to a second vaccination or challenge. In the 3 groups receiving a second dose of vaccine there was a variable SVN antibody response; group averages are shown in Figure 1. Antibodies were detectable in most pigs receiving 2 doses of vaccine lA or lC by 7 days PV, and in all these pigs by 21 days PV. The range of SVN antibody titers was from 1:2 to 1:32. There was a poor SVN antibody response in pigs receiving 2 doses of vaccine lB. Only 2 of these pigs had SVN antibodies titers by 21 days PV. The Anti-gpX ELISA did not detect gpX antibody in any pig prior to, or after vaccination.

Response to challenge

Results are summarized in Table 1. Three days after challenge exposure control pigs became depressed, had reduced appetite, and fever (104°F). These signs worsened over the next 3 days and all pigs had mucous to purulent nasal discharge, laryngitis and dyspnoea. At day 5 PC, 1 pig developed signs indicative of disturbance of the central nervous system (CNS), which included muscle trembling, incoordination, head tilting and ataxia. No control pigs died. Recovery from clinical signs began around day 7 PC and was complete by day 10 PC, except for the pig with CNS dysfunction which continued to have a head tilt throughout the trial. Clinical signs in vaccinated pigs were less severe than in controls, but present for a similar duration. No vaccinates showed

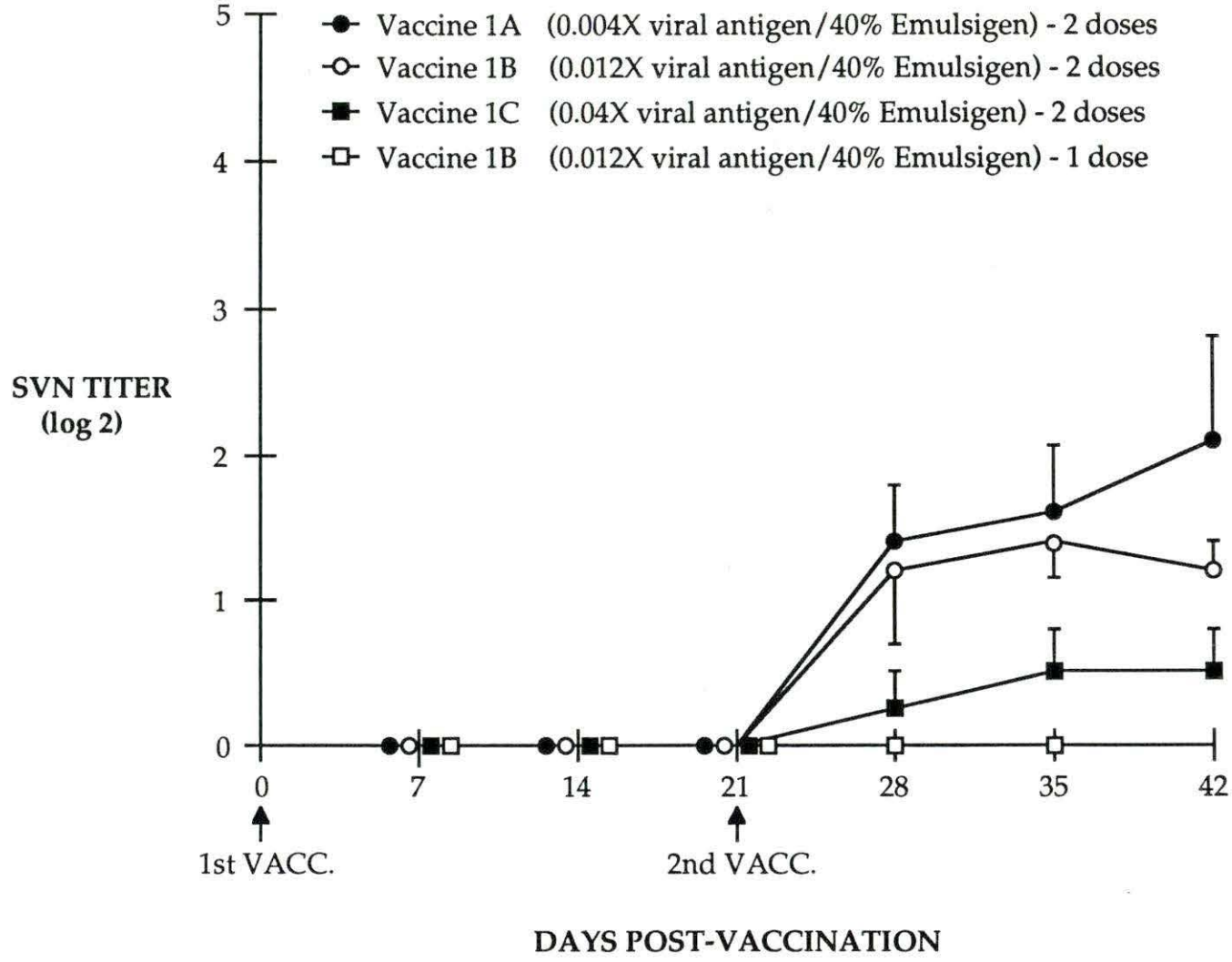

Figure 1. Serum virus neutralizing antibody response in vaccinated pigs in Trial 1. Each point is the mean ± SEM (●, ■ n = 5: ○, □ n = 4)

Table 1. Summarized serological and clinical results used to evaluate the efficacy of killed pseudorabies virus vaccines in Trial 1

Parameter		Vaccine 1A x 2	1B x 2	1C x 2	1B x 1	Control
# Challenged		5	4	5	4	5
Mean SVN antibody titer at PC day	- 0	1: 5	<2	1: 2	<2	<2
	-14	1: 675	1: 724	1: 362	1: 181	1: 48
Mean Anti-gpX ELISA reactivity at PC day	- 0	0.99	1.03	1.01	1.15	1.09
	-14	0.45	0.37	0.36	0.49	0.20
Mortality %		0	0	20	20	0
CNS dysfunct. %		0	0	0	0	20
# Shedding virus at PC day	- 3	4/5	3/4	4/5	3/4	5/5
	- 7	5/5	4/4	5/5	4/4	5/5
	-10	4/5	3/4	3/5	3/4	5/5
	-14	0/5	0/4	2/4	1/3	3/5

evidence of CNS dysfunction, although 2 died. One pig given 2 doses of vaccine lC was observed to be pale on day 10 PC and died on day 11 PC. Necropsy revealed a large gastric ulcer with hemorrhage into the stomach. Brain and tonsil samples and a nasal swab were assayed for virus but PRV was not recovered. A pig given 1 dose of vaccine lB, that had recovered by day 9 PC from the clinical signs associated with challenge, become depressed on day 12 PC and died on day 13 PC. Pseudorabies virus was recovered from the tonsil but not the brain or nasal swabs. There was no histological evidence of encephalitis in either pig.

All control pigs shed PRV from day 2 to day 11 PC, and 3 of 5 were still excreting the virus at day 14 PC. All vaccinated pigs shed virus PC, with the number of pigs shedding virus peaking around day 7 PC. Virus shedding for vaccinated pigs was of a shorter duration than for control pigs, although three vaccinates were shedding virus 14 days PC.

The rectal temperatures of vaccinated pigs were significantly lower ($P < 0.05$) than those of controls between days 3 to 7 PC, although the duration of fever was similar for vaccinates and controls.

The mean body weight of control pigs was 11 kg lower ($P < 0.05$) than the combined mean body weight for all vaccinates at 14 days PC.

All vaccines induced some degree of protection against challenge with virulent PRV. Compared with control pigs, vaccinated pigs had reduced fever, shorter periods of virus shedding, no signs of CNS dysfunction and greater body weight 14 days PC.

The SVN antibody titers of pigs rose rapidly PC, with the SVN antibody titers of vaccinated pigs being significantly higher ($P < 0.05$) than those of the

control pigs 14 days PC. All vaccinated and control pigs were postive by Anti-gpX ELISA 14 days PC.

Trial 2

Response to vaccination

One dose of the KV-SV vaccines induced SVN antibody titers in only 4 of the 15 pigs vaccinated with these vaccines. In contrast all PR-Vac®-Killed vaccinates and 4 of 5 Porci-Rab® vaccinates developed SVN antibody, although the highest SVN antibody titer was only 1:4. The group geometric mean SVN antibody titers are shown in Figure 2. At day 40 PV none of the pigs vaccinated with KV-SV vaccines had detectable gpX antibodies, whereas 5 of the 10 pigs vaccinated with the commercially available KPRV vaccines were positive by Anti-gpX ELISA.

Response to challenge

The clinical signs observed in control pigs were more severe than those seen in the control pigs in Trial 1, although the duration of clinical signs was similar. At day 8 PC, 2 control pigs developed CNS dysfunction which included muscle tremors and head tilting. Clinical signs in vaccinated pigs varied considerably between and within groups. Table 2 summarizes the results of the 6 groups. Using a subjective assessment of the clinical signs for each group as a whole, the KV-SV vaccinated groups were more severely affected by challenge than the PR-Vac®-Killed or Porci-Rab® vaccinated groups, but less affected than the control group. However, each of the KV-SV vaccinated groups had 2 pigs which developed CNS dysfunction.

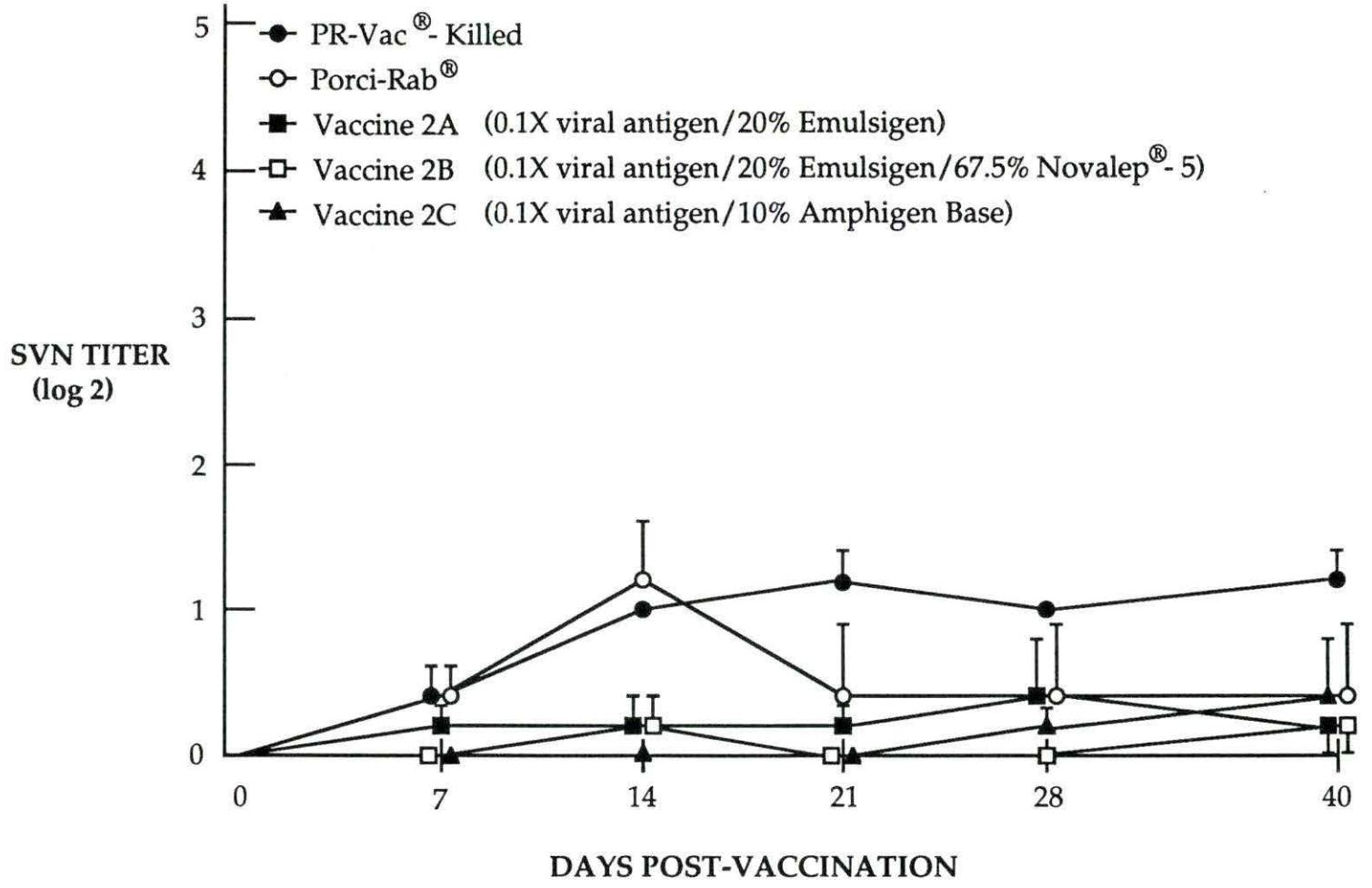

Figure 2. Serum virus neutralizing antibody response in vaccinated pigs in Trial 2. Each point is the mean ± SEM (n = 5)

Table 2. Summarized serological and clinical results used to evaluate the efficacy of killed pseudorabies virus vaccines in Trial 2

Parameter		Vaccine 2A	2B	2C	PR-Vac -Killed	Porci -Rab	Control
# Challenged		5	5	5	5	5	4
Mean SVN antibody titer at PC day	- 0	<2	<2	<2	1: 2	<2	<2
	-14	1: 215	1: 891	1: 256	1: 2352	1: 304	1: 45
Mean Anti-gpX ELISA reactivity at PC day	- 0	0.99	1.02	1.00	0.79	0.73	1.00
	-14	0.30	0.33	0.44	0.13	0.11	0.33
Mortality %		20	0	0	0	20	0
CNS dysfunct. %		40	40	40	0	20	50
# Shedding virus at PC day	- 7	3/5	3/5	4/5	0/5	3/5	4/4
	-10	0/5	0/5	0/5	0/5	1/4	2/4
	-14	0/4	0/5	0/5	0/5	0/4	2/4
Weight gain (kg) post-challenge		4.6	- 3.7	0.1	10.8	2.4	-3.2

One of these pigs which received vaccine 2A died, as did one pig in the Porci-Rab® group. At necropsy both pigs had a severe, purulent bronchopneumonia with a high population of Pasteurella multocida cultured from lung samples. Brain, tonsil, lung and spleen samples from both pigs were assayed for virus. Pseudorabies virus was recovered only from the tonsil of the pig vaccinated with Porci-Rab®. However, histologically there was evidence of moderate lymphocytic perivascular cuffing, focal gliosis and mild neuronal necrosis in the brains of both pigs, which was suggestive of viral encephalitis.

All control pigs shed virus at day 7 PC and 2 were shedding virus at day 14 PC. Vaccination prevented shedding in the PR-Vac®-Killed group by day 7 PC, and reduced the number of pigs shedding virus in the other groups. No vaccinates were shedding virus at day 14 PC.

All control pigs lost body weight and as a group had a mean body weight loss of 3.2 kg over the 14 days PC. As a group, PR-Vac®-Killed vaccinates had the highest ($P < 0.05$) mean body weight gains PC. In the other vaccinated groups there was considerable variation in body weight change within groups.

As in Trial 1 the SVN antibody titers of pigs rose rapidly PC, with the SVN antibody titers of vaccinated pigs being significantly higher ($P < 0.05$) than those of the control pigs 14 days PC. However, the presence of SVN antibody at the time of challenge of KV-SV vaccinates did not correlate with protection, as two of four pigs with SVN antibody titers developed CNS dysfunction PC. All pigs were positive by Anti-gpX ELISA 14 days PC.

Trial 3

Response to vaccination

The group geometric mean SVN antibody responses PV are shown in Figure 3. One dose of the KV-SV vaccine containing Montanide ISA 50 (vaccine 3D) induced SVN antibody titers ranging from 1:16 to 1:32 by day 26 PV. In contrast, vaccination of pigs with vaccines 3B and 3C, containing 10% Syntrogen, did not induce detectable SVN antibody titers. As in Trials 1 and 2 the SVN antibody titers of pigs vaccinated with KV-SV preparations containing Emulsigen were low and variable. Vaccinated pigs remained negative on the Anti-gpX ELISA.

Pigs receiving vaccine 3D were necropsied on day 14 PC. All pigs had lesions in the muscle at the site of vaccine injection. Grossly an area of muscle 1.5 to 3 cm in diameter was pale and firm. When the lesion was cut numerous small globules of vaccine residue were evident. Histologically each lesion contained numerous residue globules, each encapsulated in connective tissue.

Vaccination had no effect on weight gain prior to challenge.

Response to challenge

Results are summarized in Table 3. All 5 control pigs developed clinical signs typical of pseudorabies. Pigs become depressed, had reduced appetite and mucous nasal discharge on day 4 PC. These symptoms worsened by day 5 PC and all pigs showed dyspnoea. Signs indicative of CNS dysfunction developed on day 5 PC and 3 pigs had died by day 6 PC. Histologically, these three pigs showed evidence of viral encephalitis and PRV was isolated from the brain, lung, spleen and tonsil of all 3 pigs. The 2 surviving control pigs had

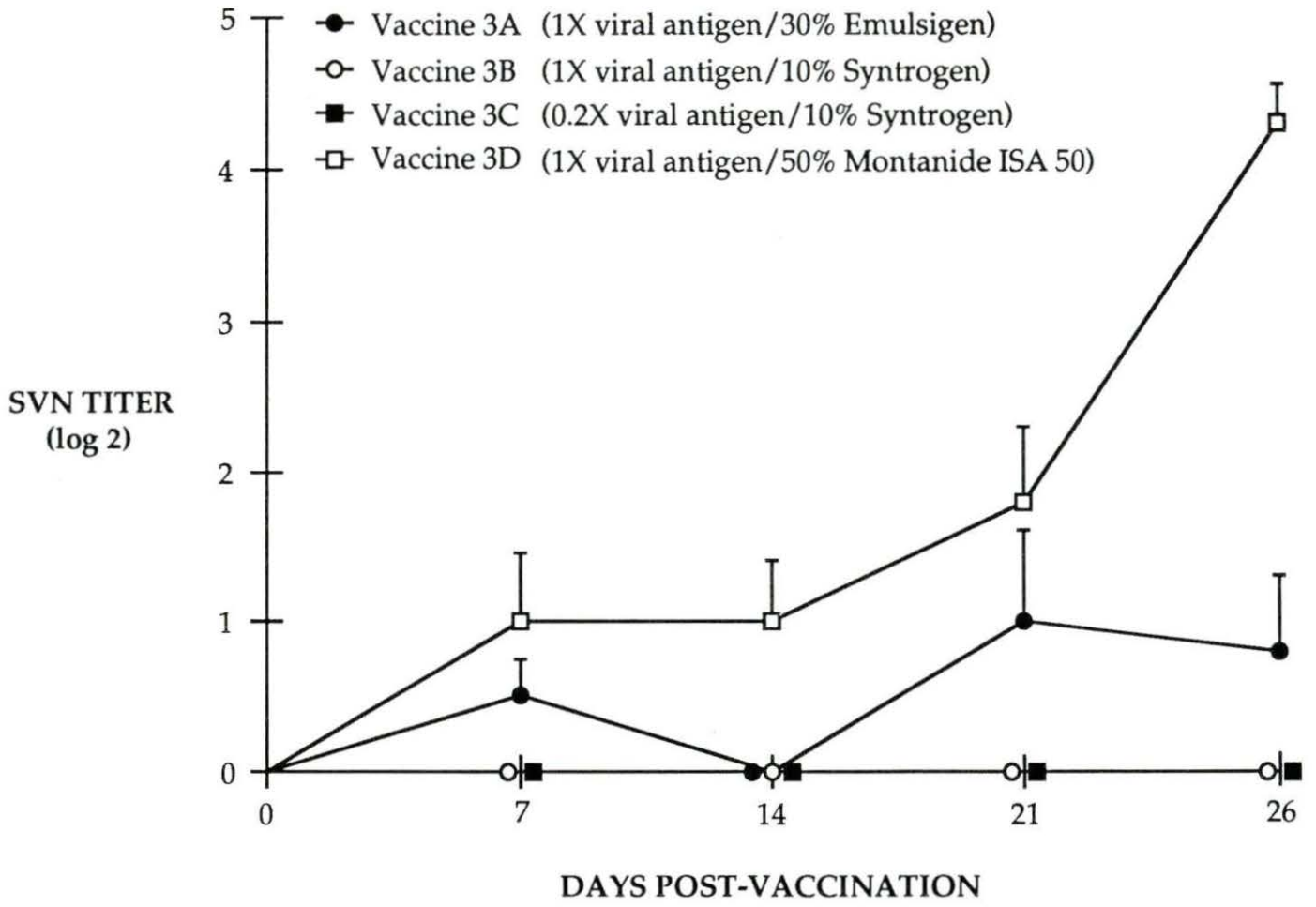

Figure 3. Serum virus neutralizing antibody response in vaccinated pigs in Trial 3. Each point is the mean ± SEM (○, ■ n = 5; ●, □ n = 4)

Table 3. Summarized serological and clinical results used to evaluate the efficacy of killed pseudorabies virus vaccines in Trial 3

Parameter		Vaccine 3A	3B	3C	3D	Control
# Challenged		4	5	5	4	5
Mean SVN antibody titer at PC day	- 0	<2	<2	1: 2	1: 20	<2
	-14	1: 16	1: 56	1: 14	1: 2048	1: 8
Mean Anti-gpX ELISA reactivity at PC day	- 0	0.91	0.88	0.93	0.85	0.96
	-14	0.51	0.23	0.16	0.44	0.49
Mortality %		50	0	0	0	60
CNS dysfunct. %		50	0	0	0	100
# Shedding virus at PC day	- 3	4/4	4/5	5/5	3/4	5/5
	- 6	1/4	1/5	0/5	0/4	2/2
	- 9	0/2	0/5	0/5	0/4	2/2
	-12	0/2	0/5	0/5	0/4	1/2
Weight gain (kg) pre-challenge		11.1	11.6	12.6	13.2	15.0
Weight gain (kg) post-challenge		5.8	5.4	5.6	9.0	-1.2

muscle tremor, incoordination, head tilting, and convulsions. These 2 pigs began recovering by day 8 PC, but they continued to have a head tilt until the end of the trial. Pigs receiving vaccine 3D showed the least clinical signs PC. These pigs had a slight decrease in appetite and were mildly depressed on day 5 PC. Clinical signs persisted from day 5 to 7 PC in pigs receiving vaccine 3B and 3C and they were of similar magnitude to those seen with vaccine 3D. The response of pigs receiving vaccine 3A was variable. Two pigs showed mild clinical signs between days 5 and 7 PC, whereas, the other 2 pigs became extremely depressed, developed CNS dysfunction and died by day 7 PC. Both the pigs had histological evidence of a viral encephalitis but PRV was not isolated from either brain.

Vaccination markedly decreased the duration of virus shedding PC. Both surviving controls shed virus on day 9 PC, whereas, pigs vaccinated with vaccine 3C or 3D did not shed virus after day 3 PC and only 1 pig in each group receiving vaccine 3A or 3B shed virus at day 6 PC.

Pigs receiving vaccine 3D had the least severe clinical signs and gained significantly ($P < 0.05$) more weight than pigs in the other treatment groups.

Vaccinated pigs had a rapid rise in SVN antibody titers PC. Those vaccinates that had no detectable SVN antibody response to vaccination still had an anamnestic rise in SVN antibody PC, and the pigs vaccinated with vaccine 3B or 3C were well protected from challenge. One pig receiving vaccine 3A had an SVN antibody titer of 1:4 at the time of challenge but was not protected.

Trial 4

Response to vaccination

The group geometric mean SVN antibody responses PV are shown in Figure 4. One dose of the KV-SV vaccines containing Montanide ISA 50 induced SVN antibody titers ranging from 1:4 to 1:32 by day 22 PV. There was no evidence to suggest that a change in viral antigen concentration from 1X to 0.2X/dose had any effect on the SVN antibody response in vaccinated pigs. All pigs receiving vaccine 4D (20% Syntrogen) had SVN antibody titers of 1:2 by day 22 PV. Vaccinated pigs remained negative on the Anti-gpX ELISA prior to challenge.

Intramuscular swelling at the site of injection was detected by palpation in 2 pigs in each of groups 4A, 4C and 4D and 1 pig in group 4B at day 7 PV. At day 21 PV, reactions could still be palpated in 4 pigs that had received vaccines containing Montanide ISA 50, but by day 36 PV no reactions were detected by palpation.

Vaccination had no apparent effect on weight gains prior to challenge.

Response to challenge

Results are summarized in Table 4. All control pigs developed clinical signs typical of pseudorabies. Signs indicative of CNS dysfunction were evident in all pigs by day 6 PC. The severity of signs varied from trembling and slight incoordination in 1 pig, to ataxia, head tilting, and convulsions in the 2 most severely affected pigs. All control pigs survived and began recovering by day 8 PC although 2 pigs continued to head tilt until the end of the trial. Only 2 vaccinated pigs showed any clinical signs in the 14 days PC. One pig receiving vaccine 4A was depressed, trembled and circled aimlessly on day 9 PC. The

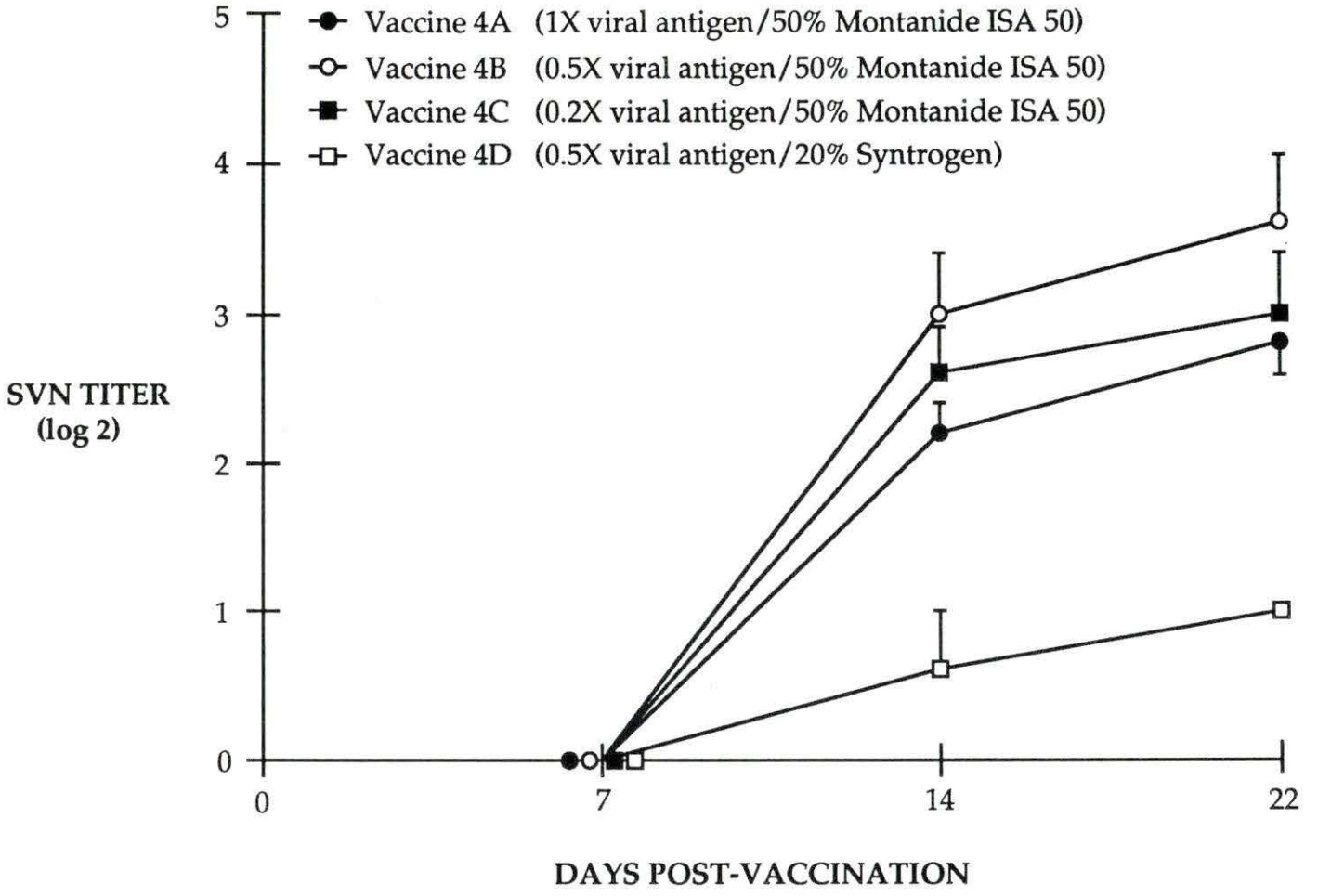

Figure 4. Serum virus neutralizing antibody response in vaccinated pigs in Trial 4. Each point is the mean ± SEM (n = 5)

Table 4. Summarized serological and clinical results used to evaluate the efficacy of killed pseudorabies virus vaccines in Trial 4

Parameter		Vaccine 4A	4B	4C	4D	Control
# Challenged		5	5	5	5	5
Mean SVN antibody titer at PC day	- 0	1: 7	1: 12	1: 8	1: 2	<2
	-14	1: 776	1: 1176	1: 1783	1: 294	1: 24
Mean Anti-gpX ELISA reactivity at PC day	- 0	0.95	0.92	0.97	1.07	0.94
	-14	0.80	0.45	0.37	0.38	0.30
Mortality %		0	0	0	0	0
CNS dysfunct. %		20	0	0	0	100
# Shedding virus at PC day	- 3	0/5	0/5	1/5	5/5	5/5
	- 6	0/5	0/5	0/5	0/5	5/5
	- 9	0/5	0/5	0/5	0/5	5/5
	-12	0/5	0/5	0/5	0/5	4/5
Weight gain (kg) pre-challenge		7.0	8.4	8.7	8.4	7.7
Weight gain (kg) post-challenge		5.5	7.0	6.2	5.3	0.6

signs of CNS dysfunction were absent by day 10 PC and the pig had recovered by day 12 PC. Necropsy on day 14 PC revealed no gross abnormalities. Histologically there was lymphocytic perivascular cuffing in the brain suggestive of a viral encephalitis,however, PRV was not isolated from the brain. One pig receiving vaccine 4D was slightly depressed on days 6 and 7 PC but showed no other clinical signs.

Virus was shed by all control pigs on days 3, 6, and 9 PC and 4 of the 5 controls on day 12 PC. In contrast, PRV was only isolated from vaccinated pigs on day 3 PC and this was limited to the 5 pigs receiving vaccine 4D and 1 pig receiving vaccine 4C.

Control pigs as a group had only a minimal gain in body weight in the 14 days PC. The weight gains of vaccinated pigs were significantly ($P < 0.05$) higher than those of control pigs and there was no evidence to suggest there were any differences in weight gain between vaccinated groups.

The timing of the anamnestic SVN antibody response varied between vaccinated pigs. At day 7 PC there was at least a 4-fold increase in SVN antibody titers in all 5 pigs receiving vaccine 4D (20% Syntrogen). The SVN antibody response in pigs receiving Montanide ISA 50 vaccines ranged from no change in 4 pigs up to a change similar to that seen in the Syntrogen vaccinates. By day 14 PC the group mean SVN antibody titers of groups 4A,4B,and4C were similar and much higher than that of the Syntrogen group. Four pigs all in group 4A (1X viral antigen/50% Montanide ISA 50), remained negative by the Anti-gpX ELISA at day 14 PC, although there was evidence to suggest low levels of antibodies to gpX were present in the serums of these pigs. The slow anamnestic SVN antibody response, the low levels of gpX antibody detected,

and the failure to isolate the challenge virus from these pigs suggests there was limited replication of PRV in these pigs PC. At day 21 PC, 3 of these 4 pigs were positive by the Anti-gpX ELISA.

DISCUSSION

The SVN antibody response by pigs after IM inoculation with vaccines containing KV-SV was more dependent on the type and concentration of adjuvant than on the amount of virus antigen used in each preparation.

Vaccines adjuvanted with Montanide ISA 50 induced significantly ($P < 0.05$) higher SVN antibody titers than vaccines containing other adjuvants. In Trial 3, vaccines containing Emulsigen or Syntrogen induced group geometric mean SVN antibody titers of <1:2 by day 26 PV, whereas the Montanide ISA 50 vaccine containing the same amount of PRV antigen induced a group geometric mean SVN antibody titer of 1:20. The vaccine in Trial 2 containing Amphigen Base also induced a poor SVN antibody response.

It was possible to alter the immunogenicity of vaccines by varying the concentration of adjuvant. In Trial 3, vaccines containing 10% Syntrogen did not induce detectable SVN antibody, whereas the 5 pigs in Trial 4 receiving vaccine 4D, containing twice the concentration of this adjuvant, were all positive by the SVN test at day 22 PV.

Varying the PRV antigen concentration of KV-SV vaccines had only a slight effect on the induction of a SVN antibody in vaccinated pigs. In Trial 3 a 2 $\log_{10}$ increase in pre-inactivation infectivity titer of Emulsigen vaccines caused a variable increase in SVN antibody titers. In Trial 4 a reduction in infectivity titer by 0.7 $\log_{10}$/dose of Montanide ISA 50 vaccines caused no change in SVN antibody response at day 22 PV. There may not be a linear relationship between infectivity titers and PRV antigen concentration of the inactivated vaccines, as

the total PRV antigen present may include defective virus particles and debris from infected cells, in addition to infective virus particles.

Vaccination with PR-Vac®-Killed or Porci-Rab® induced low and variable SVN antibody titers in pigs. In addition, 50% of these vaccinates were positive by the Anti-gpX ELISA. In contrast, all pigs vaccinated with KV-SV vaccines remained negative by the Anti-gpX ELISA and there was no evidence to suggest there was any difference in the Anti-gpX ELISA reactivity between non-vaccinated and vaccinated pigs. Therefore, the Anti-gpX ELISA could be used in conjunction with KV-SV vaccines to differentiate PRV vaccine induced antibodies from antibodies induced by natural exposure.

The SVN antibody titer induced by vaccination has little predictive value on the degree of protective immunity provided by PRV vaccines.[4] Vaccine efficacy is best evaluated by comparing the effects that challenge exposure to virus has on vaccinated and control animals under standard laboratory conditions. By measuring mortality, the development of CNS dysfunction, weight gains, fever and virus shedding in vaccinated pigs, PRV vaccines can be effectively compared.[4] If vaccines are tested at different times, meaningful comparisons can still be made if a standard response in control pigs is achieved. For PRV vaccines, a challenge dose of virus sufficient to cause CNS dysfunction in 80% of control pigs is desired. The severity of clinical signs following challenge exposure depends on the virulence of the viral strain, the age of the pigs, dose of virus, and route of exposure.[9] There have been no previous reports on the use of PRV strain VDL 4892 for challenge exposure of pigs. Therefore, the dose of challenge virus used initially was based on reports of the use of other PRV strains to challenge pigs intranasally.[1,4,9,14] The dose of virus

was changed with successive trials until an acceptable dose for the age of pigs being used was obtained in Trials 3 and 4.

The KV-SV vaccines containing Montanide ISA 50 and Syntrogen conferred good protection against challenge with PRV strain VDL 4892. Although, the depression and reduced appetite seen in some pigs PC indicates that all vaccinates were not completely protected. Virus shedding from vaccinated pigs PC was of much shorter duration than from control pigs. Virus shedding PC by pigs vaccinated with commercially available PRV vaccines has been reported to last up to 8 days PC.[3] The duration of shedding by pigs vaccinated with Montanide ISA 50 or Syntrogen KV-SV vaccines compares favorably with this. In Trial 4, virus shedding could be detected in only 1 pig receiving a Montanide ISA 50/KV-SV vaccine and no vaccinates shed virus after day 3 PC.

Vaccination had pronounced effects on the growth rate of pigs PC. Pigs receiving PR-Vac®-Killed or KV-SV vaccines containing Montanide ISA 50 or 20% Syntrogen had comparable growth rates that were significantly ($P < 0.05$) higher than growth rates of control pigs. Vaccines containing a lower concentration of Syntrogen were slightly less effective. The development of body weight PC is considered an important parameter for evaluating vaccine efficacy because it is an objective indicator of severity of illness and is directly related to economic losses.[4] This parameter was used for vaccine evalution in Trials 2, 3 and 4, in preference to period of fever, because it revealed the most pronounced differences between vaccinated and control pigs.

As the mortality, percent of pigs with CNS dysfunction and weight gains of control pigs PC varied between trials, inter-trial comparisons of vaccine

efficacy needs to be done with care. Nevertheless, it seems reasonable to group the KV-SV vaccines into 3 categories according to their levels of efficacy: (i) the least effective were vaccines containing Emulsigen or Amphigen Base (vaccines 1A, 1B, 1C, 2A, 2B, 2C and 3A); (ii) vaccines containing 10% Syntrogen (vaccines 3B and 3C) were of moderate efficacy, (iii) vaccines containing Montanide ISA 50 (vaccines 3D, 4A, 4B and 4C) or 20% Syntrogen (vaccine 4D) gave the best protection. The 2 commercially available vaccines used in Trial 2, PR-Vac® Killed and Porci-Rab® would be in categories (iii) and (i) respectively.

The efficacy of vaccines 3D, 4A, 4B, 4C and 4D would make them suitable for use as killed vaccines against pseudorabies. However, factors such as cost, safety, stabilitiy and licensing difficulties need to be considered prior to making a decision on which vaccine would be best for commercial production.[18] Adjuvants added to veterinary vaccines must meet standards of purity, be nontoxic, not denature specific substances in the product through the dating period, and also not leave harmful residues in meat.[7]

Montanide ISA 50 is an an oil based adjuvant. The immunoenhancing effects of oil are related to its protection of antigens from host degradation, the transport of antigens through the lymphatic system where foci of antibody production can be established, and the progressive release of antigens from the site of injection.[15] The manufacturer of Montanide ISA 50 reports that the product stimulates the immune response to many viral antigens and that foot-and-mouth vaccines containing Montanide ISA have been used successfully in South America.[16] However, there are no reports of its use in USDA licensed vaccines. One disadvantage identified in the study was the IM reaction at the site of injection. This localized reaction consisting of fibrous tissue containing

small cyst-like spaces filled with emulsion is not uncommon with oil adjuvants but may be an obstacle to USDA licensing. One other disadvantage of the vaccines adjuvanted with Montanide was that they were viscous and difficult to inject through an 18 gauge needle. A major advantage of vaccines 3D, 4A, 4B and 4C (50% Montanide ISA 50) over vaccine 4D (20% Syntrogen) was that they induced much higher SVN antibody titers. Vaccines that induce high antibody levels in the colostrum of sows, so that newborn piglets receive high maternal immunity, may be preferable.

Finally, the economic aspects are an important determinate on the choice of PRV vaccines. The relative costs of vaccine production with Montanide ISA 50 and Syntrogen would need to be evaluated prior to selecting which of the KV-SV vaccines was best suited for commercial production.

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SECTION II. EVALUATION OF THE PSEUDORABIES ANTI - GPX ELISA TEST

SUMMARY

The persistence of antibodies to glycoprotein X (gpX) in the serum of pigs experimentally infected with pseudorabies virus (PRV) was determined using the HerdChek®: Anti-PRV-gpX assay (Anti-gpX ELISA). Antibodies to gpX were detected for at least 365 days post-challenge in non-vaccinated pigs. Previous sensitization of pigs by vaccination with PRV/Marker® had no apparent effect on the antibody response of pigs to gpX post-challenge. In determining previous exposure of pigs to PRV strains containing the gpX gene, the Anti-gpX ELISA was highly specific, but its sensitivity was lower than the standard serological procedures currently used for detecting PRV antibodies.

INTRODUCTION

The move toward a national pseudorabies eradication program has dictated a need for improved diagnostic tests. Of particular importance has been the need for a diagnostic serology test that would differentiate vaccinated pigs from infected pigs. Vaccination of pigs with pseudorabies virus (PRV) vaccines is commonly practiced. Vaccination will reduce mortalities, shorten growth arrest periods, and decrease the shedding of virulent PRV after challenge.[1] Vaccination will not prevent infection or the establishment of latent infection with virulent virus.[7] Therefore, PRV exposure following vaccination needs to be assessed, but, standard serological procedures are of limited value since they cannot distinguish antibodies induced by natural infection from those induced by PRV vaccines. Considerable effort has been directed at this problem as the inability to differentiate these antibody responses markedly decreases the efficiency of eradication programs utilizing vaccination. The creation of gene deleted vaccines and their associated diagnostic tests are major breakthroughs in overcoming the problem with conventional vaccines and standard serological procedures.

Genetically engineered PRV vaccines have deletions of non-essential genes. One example is the deletion of the gene coding for glycoprotein X (gpX), which is secreted by PRV infected cells. Glycoprotein X is a major antigen interacting with an infected pig's immune system.[4] However, antibodies to gpX have no PRV neutralizing activity so the deletion of the gpX gene does not reduce the efficacy of the vaccine.[5] Two USDA licensed gpX deleted vaccines

are available, PRV/Marker®[a] and Tolvid®[b]. Both vaccine strains also have the gene coding for thymidine kinase (TK) deleted from their genomes. The deletion of the TK gene reduces vaccine virulence and the likelihood of the establishment of latent infection with the vaccine strain.[8]

PRV/Marker® has an accompanying USDA licensed diagnostic test, the HerdChek®: Anti-PRV-gpX[c] (Anti-gpX ELISA), which is specific for antibodies to gpX in pig serum.[3] It ignores antibody titers in animals vaccinated with PRV/Marker®, but detects antibody in animals infected with field strains or vaccinated with strains that contain the gpX gene.

The major objectives of this study were to determine the persistence of antibodies to gpX in non-vaccinated and PRV/Marker® vaccinated pigs following PRV infection, and to compare the Anti-gpX ELISA with the standard serological procedures used to assess PRV exposure.

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[a] SyntroVet Incorporated, Lenexa, Kansas.
[b] The Upjohn Company, Kalamazoo, Michigan.
[c] IDEXX Incorporated, Portland, Maine.

MATERIALS AND METHODS

Trial I

Four 8 week old pigs from a PRV negative herd were challenged intranasally with $10^{5.0}$ plaque forming units (PFU) of the Shope strain of PRV. Serums were collected on the day of challenge and 48 times over the next 365 days.

Trial II

Three groups of 5 pigs, 6 to 8 weeks of age, from a PRV negative herd were used. Group A remained non-vaccinated controls. Group B received one dose of PRV/Marker® intramuscularly (IM) according to the manufacturers instructions. Group C received 10 times the manufacturers recommended dose of PRV/Marker® IM. All pigs were challenged intranasally with $10^{3.0}$ PFU of a virulent strain of PRV, VDL 4892, 10 weeks post-vaccination (PV). Serums were collected from all pigs on the day of vaccination, days 14, 62, and 66 PV, the day of challenge, and days 3, 7, 10, 14, 21 and 28 post-challenge (PC). To determine the persistence of antibodies to PRV, Group B was also bled on days 42, 53, 67, and 115 PC.

Test Serums

A total of 1,372 pig serums were assayed. These were divided into 6 groups. Included in these groups were pig serums utilized in Trials I and II.

Group 1 Serums from 1,017 non-vaccinated pigs originating from PRV negative herds (no clinical or serological evidence of PRV infection).

Group 2 Serums from 157 pigs vaccinated with 1 or 2 doses of PRV/Marker® and bled 3 weeks PV.

Group 3 Serums from 35 pigs vaccinated with 1 or 2 doses of Tolvid® and bled 3 weeks PV.

Group 4 Serums from 60 pigs vaccinated with killed or modified live gpX positive PRV vaccines and bled 3 weeks PV.

Group 5 Serums from 48 pigs infected intranasally with PRV strain VDL 4892, and bled 2 to 4 weeks PC.

Group 6 Serums with varying virus neutralizing antibody titers from 55 non-vaccinated pigs from PRV infected herds.

Assays

Serums were tested with the Anti-gpX ELISA, the HerdChek®: Anti-PRV (S)[c] (Anti-PRV ELISA), the serum virus neutralization (SVN) test, and by radioimmunoprecipitation (RIP).

The Anti-gpX ELISA is a competitive enzyme immunoassay, utilizing monoclonal antibodies that are specific for gpX. The manufacturer describes the principles of the test in the kit insert[3] as follows: The Anti-gpX ELISA is performed in a PRV antigen coated microwell using a two-fold (1:2) serum dilution. During the first incubation, PRV antibodies present in the serum, including those produced against gpX, react with antigens on the plastic. Subsequent to a wash step, an anti-gpX monoclonal antibody conjugate is added to the microwell and is allowed to compete for the gpX viral antigen during a second incubation. If no gpX antibodies are present in the test serums, the conjugated gpX antibodies are free to react with the gpX antigen. Conversely, if

gpX antibodies are present in the test serum, the enzyme-conjugated monoclonal antibodies are blocked from reacting with the antigen. Following this incubation period, the unreacted conjugate is removed by washing and a substrate/chromagen solution is added. In the presence of enzyme, the substrate is converted to a product which reacts with the chromophore to generate a blue color. The absorbance at 650 nm A(650), is measured by a spectrophotometer. Results are calculated by dividing the A(650) of the sample by the mean A(650) of the negative control, resulting in a S/N value. The quantity of antibodies to gpX is inversely proportional to the A(650) and, thus, to the S/N value. If the S/N is less than or equal to 0.70, the test is classified as positive for antibodies to the gpX antigen of PRV. If the S/N is greater than 0.70 the test is classified negative. The presence of PRV antibodies, including anti-gpX, indicates a previous exposure to a field strain of PRV, or application of conventionally modified live or killed virus vaccines. The presence of PRV antibodies detected by the Anti-PRV ELISA and/or SVN test, but absence of antibodies to gpX antigen as assessed by the Anti-gpX ELISA indicates a response to a gpX deleted vaccine.

The Anti-PRV ELISA is a commercially available indirect enzyme immunoassay used as a screening test for PRV antibodies.[9] The presence, or absence, of antibody is determined by calculating the sample absorbance to weak positive absorbance (S/P) ratio. Serum samples with S/P ratios of less than 0.40 are classified negative for PRV antibodies. If the S/P ratio is greater than or equal to 0.40 the sample is classified positive for PRV antibodies.

Serum virus neutralization titers were determined as described by Hill et al.[2] The SVN test is the standard serological test used to detect antibody to PRV.

Radioimmunoprecipitation using (35S) methionine-labelled subunit diagnostic antigen (SUDA) combined with selected serums was performed as described by McGinley and Platt.[6]

RESULTS

Trial I

Prior to challenge, the 4 pigs were serologically negative by Anti-PRV ELISA and Anti-gpX ELISA. After challenge the pigs developed positive reactions to both tests. Group Anti-PRV ELISA values were above the positive threshold by day 8 PC and peaked at day 280 PC (Figure 1). Anti-gpX ELISA values were positive by day 8 PC and peaked at day 300 PC (Figure 2). Considerable variation in antibody response was seen between individual pigs, despite being from the same litter. However, all pigs remained positive on both tests from day 8 to day 365 PC.

Trial II

All 15 pigs were serologically negative for PRV antibodies at the commencement of the trial. Anti-PRV ELISA values for pigs in Groups B and C were positive 14 days PV and remained positive throughout the trial. Anti-gpX ELISA values remained negative PV and pre-challenge. The group mean Anti-PRV ELISA and Anti-gpX ELISA values from day 0 to 28 PC are shown in Table I. All control pigs were Anti-PRV ELISA and Anti-gpX ELISA positive from day 10 to 28 PC. The Anti-gpX ELISA values for all vaccinates were positive by day 21 PC and remained positive until day 115 PC for the 5 pigs in Group B. The mean Anti-gpX ELISA values for Group B are shown in Figure 2. Anti-PRV ELISA values for vaccinates increased PC and peaked at day 28 PC. The mean values for Group B are shown in Figure 1. There was little variation in Anti-PRV ELISA or Anti-gpX ELISA reactivity in non-vaccinated pigs, however,

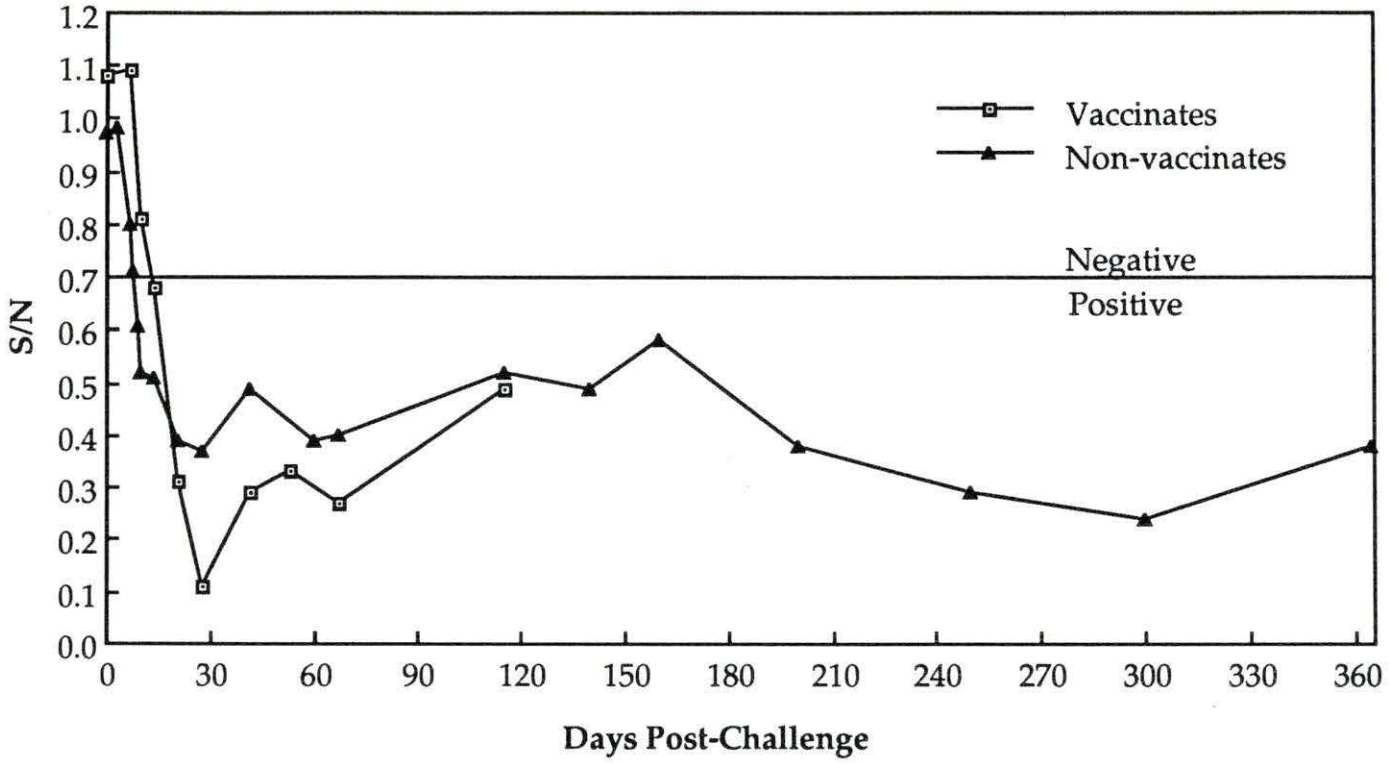

Figure 1. Development and persistence of anti-gpX antibody in non-vaccinated and PRV/Marker® vaccinated pigs following intranasal inoculation with PRV, as assessed by the Anti-gpX ELISA.

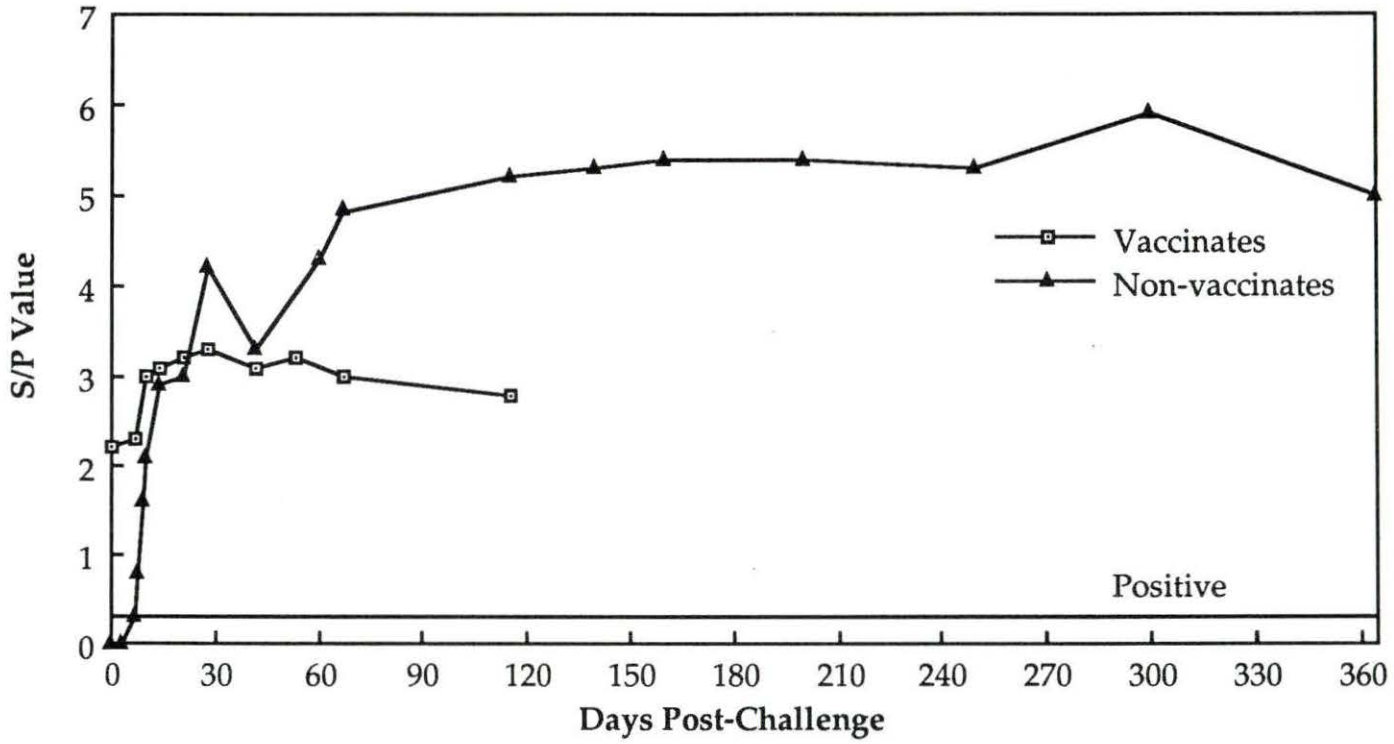

Figure 2. Development and persistence of anti-PRV antibody in non-vaccinated and PRV/Marker® vaccinated pigs following intranasal inoculation with PRV, as assessed by the Anti-PRV ELISA.

Table 1. Anti-gpX ELISA and Anti-PRV ELISA group mean reactivities from day 0 to day 28 post-challenge for the groups in Trial II

Day post-challenge	Group A non-vaccinates		Group B 1 x PRV/Marker®		Group C 10 x PRV/Marker®	
	gpX[a]	PRV[b]	gpX	PRV	gpX	PRV
0	1.08	0.03	0.92	2.31	1.10	2.18
3	1.20	0.00	0.99	2.25	1.06	1.94
7	1.09	0.05	1.19	2.45	0.84	2.83
10	0.40	1.30	0.81	3.16	0.83	3.25
14	0.19	2.11	0.68	3.29	0.30	3.05
21	0.26	2.61	0.31	3.31	0.26	3.29
28	0.07	3.01	0.11	3.33	0.18	3.15

[a] Anti-gpX ELISA S/N ratios > 0.70 are negative.
[b] Anti-PRV ELISA S/P ratios > 0.40 are positive.

reactivity in non-vaccinated pigs, however, vaccinated pigs with similar Anti-PRV ELISA reactivity had considerable variation in Anti-gpX ELISA reactivity PC.

Evaluation of Serological Tests

The distribution of Anti-PRV ELISA and Anti-gpX ELISA results in the 6 groups is shown in Table 2. Pigs in Group 1 (non-vaccinated, non-infected) were classified as negative by both assays with a high degree of accuracy. Antibodies to gpX were detected in none of the PRV/Marker® vaccinated pigs and one of the Tolvid® vaccinated pigs 3 weeks PV, whereas, all of these gpX deleted vaccinated pigs tested were positive by the Anti-PRV ELISA. The Anti-gpX ELISA was less sensitive than the Anti-PRV ELISA at detecting pigs exposed to gpX positive strains of PRV either by vaccination, experimental intranasal inoculation, or by natural infection. Twenty-five of 141 pigs positive by Anti-PRV ELISA were negative by Anti-gpX ELISA These 25 pigs had Anti-PRV ELISA S/P ratios ranging from 0.84 to 3.22. Fifteen of the pigs had SVN titers <2 and the other 10 pigs had titers of either 1:2 or 1:4.

The overall distributions of Anti-gpX ELISA reactivity for Group 1 and Groups 2 and 5 are shown in Figures 3 and 4 respectively. The S/N values of non-vaccinated pigs (mean = 0.98, standard deviation = 0.12) were significantly ($P < 0.05$) higher than those of PRV/Marker® vaccinated pigs (mean = 0.95, standard deviation = 0.10). However the means of both groups were well above the positive threshold and there was little deviation from mean reactivity in either group.

Radioimmunoprecipitation was performed on pooled serum samples of pigs in Group 1, Group 2 and Group 3 and on the serum sample in Group 3 reacting on the Anti-gpX ELISA. The SUDA did not react with antibodies in any of the serums tested, but did react with antibodies in control serum from PRV infected pigs. The monoclonal antibody to gpX used in the Anti-gpX ELISA also reacted with SUDA suggesting SUDA is the same as gpX.

Table 2. Results of Anti-gpX ELISA and Anti-PRV ELISA on test serums

Group	Frequency of Anti-gpX ELISA results		Frequency of Anti-PRV ELISA results	
	neg	pos	neg	pos
Group 1 (non-vaccinated)	1015	2	912	0
Group 2 (PRV/Marker®)	157	0	0	90
Group 3 (Tolvid®)	34	1	0	35
Group 4 (conventional vaccine)	33	17	12	38
Group 5 (PRV challenge)	1	47	0	48
Group 6 (infected herd)	3	52	0	55

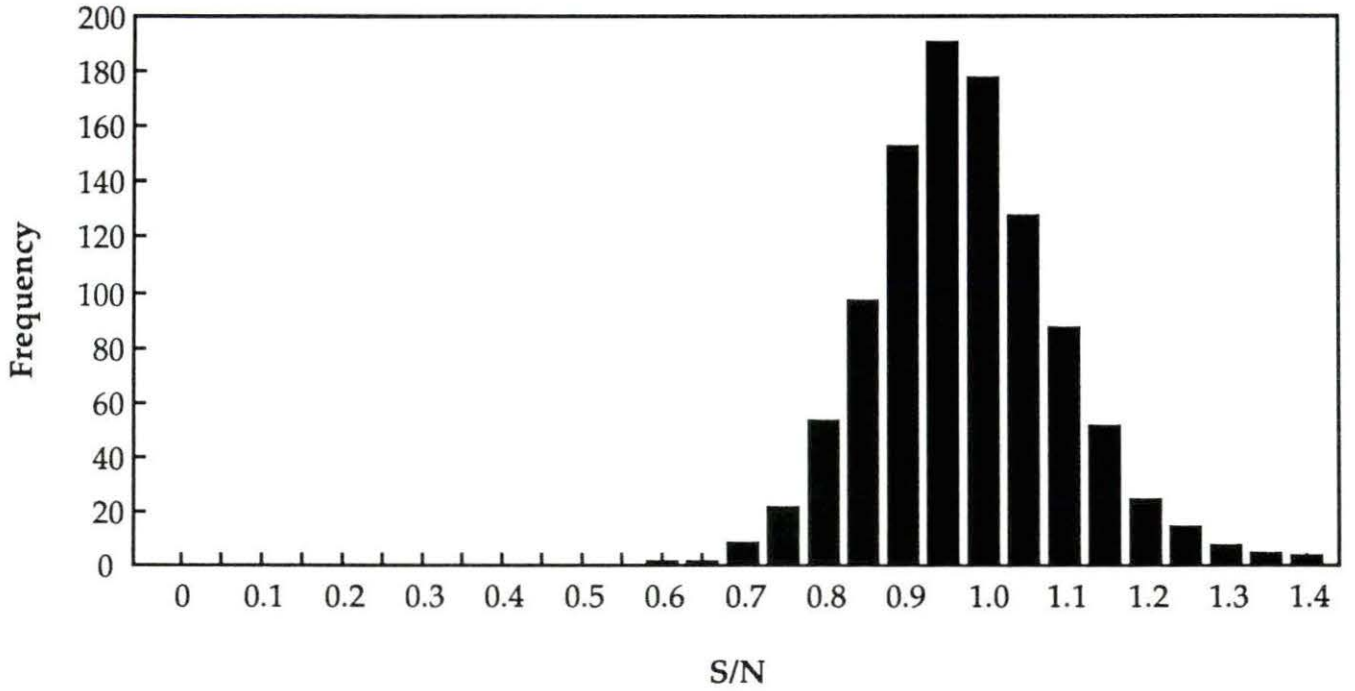

Figure 3. Distribution of Anti-gpX ELISA S/N ratios amongst 1017 serums from PRV free herds. Serums with S/N ratios > 0.70 are classified as negative for anti-gpX antibody

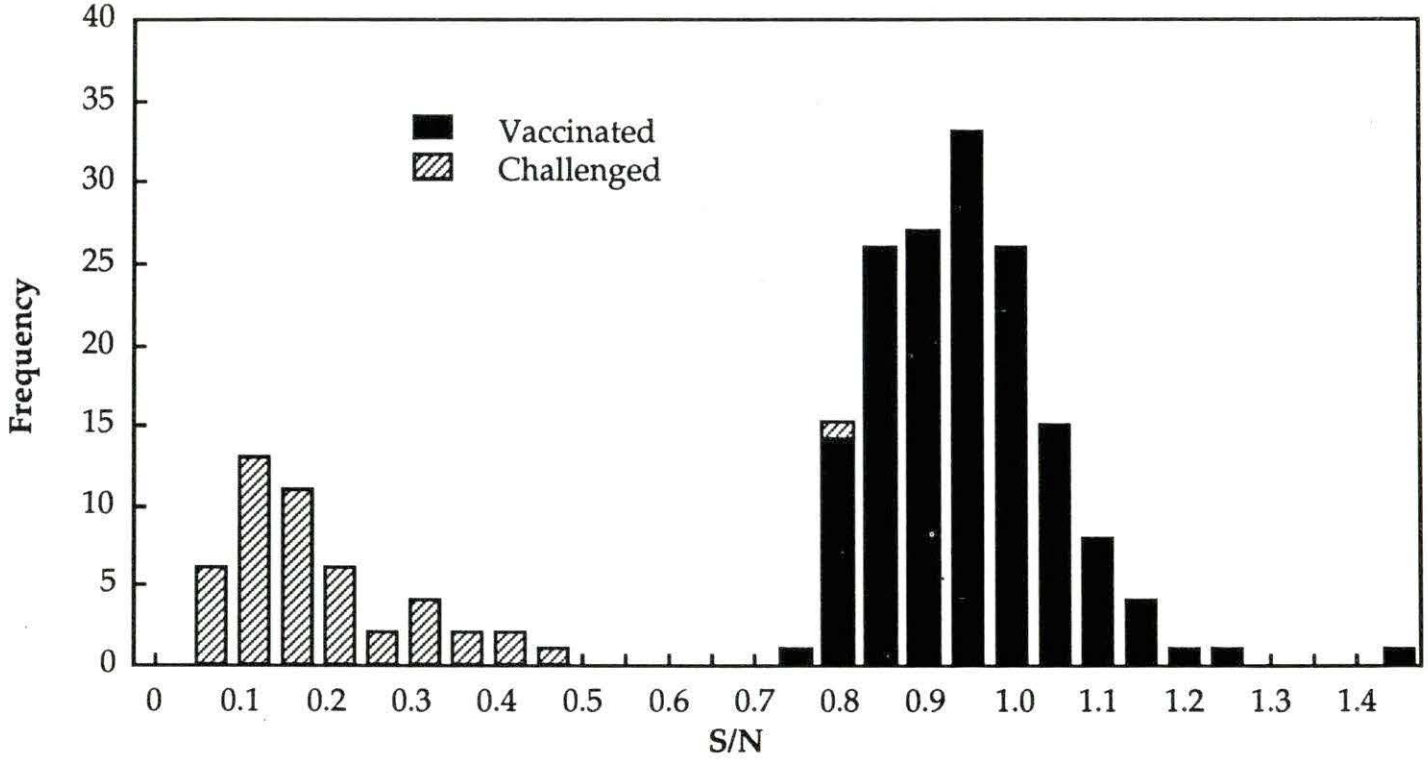

Figure 4. Distribution of Anti-gpX ELISA S/N ratios amongst 157 PRV/Marker® vaccinated pigs. Serums with S/N ratios > 0.70 are classified as negative for anti-gpX antibody

DISCUSSION

Using a S/N ratio of 0.70 as the positive/negative threshold, the Anti-gpX ELISA is highly specific for evaluating the exposure of pigs to PRV strains coding for gpX. All pigs vaccinated with PRV/Marker® and 34 of 35 pigs vaccinated with Tolvid® were negative on this test. The 1 pig vaccinated with Tolvid® that was positive by Anti-gpX ELISA was negative by RIP. This suggests the gpX antibodies were not present in the serum of this pig and the the positive reaction on the Anti-gpX ELISA was due to non-specific competition with the conjugated monoclonal antibody to gpX. The lower S/N values for PRV/Marker® vaccinated pigs as compared to non-vaccinated pigs does not appear to be due to the production of gpX antibodies by PRV/Marker® vaccinates. A second vaccination with PRV/Marker® did not induce an anamnestic response detectable by the Anti-gpX ELISA, and pooled serums from PRV/Marker® vaccinated pigs were negative by RIP. It is likely that the binding of other PRV antibodies to the PRV antigen coated microwell caused slight steric hindrance to the binding of the conjugated anti-gpX monoclonal antibody. Regardless, PRV/Marker® serums were found to show relatively little deviation from mean reactivity and were well separated from the S/N ratios found among serums from pigs tested 2 to 4 weeks after PRV challenge. As all field strains of PRV tested code for gpX^{10}, the Anti-gpX ELISA can effectively distinguish between naturally infected and vaccinated pigs if a gpX deleted vaccine has been used. However, currently the Anti-gpX ELISA can officially only be used to differentiate animals vaccinated with PRV/Marker®

because the license for a diagnostic test is restricted for use with its companion vaccine.

The ELISA discrimination endpoint chosen to distinguish positive and negative serums requires a compromise between an assay's sensitivity and specificity. The high specificity of the Anti-gpX ELISA was associated with some loss of sensitivity as compared to the Anti-PRV ELISA. This lower sensitivity means the Anti-gpX ELISA would be less effective than the Anti-PRV ELISA as a screening test for detection of antibody to PRV in pig serums. However, when used as a monitoring test subsequent to using a gpX deleted vaccine, the Anti-gpX ELISA is superior to the Anti-PRV ELISA which is of limited value in assessing PRV infection following vaccination, since it cannot distinguish between naturally infected and vaccinated pigs.

For the Anti-gpX ELISA to be an effective assay it needs to detect PRV antibodies in PRV exposed pigs, both non-vaccinated and vaccinated, for extended periods. This study demonstrated that positive levels of gpX antibody are detectable for at least 365 days and 115 days PC, in non-vaccinated and PRV/Marker® vaccinated pigs respectively. The sensitization of vaccinates with a live PRV gpX deleted vaccine did not significantly alter a pig's humoral immune response to gpX when exposed to wild type PRV. Antibodies to gpX were detectable earlier PC in non-vaccinates, probably as a result of greater viral replication in these pigs. However, by 21 days PC all vaccinates were positive by Anti-gpX ELISA and the level of antibody response was similar between groups. In Trial II the greater variation in Anti-gpX ELISA reactivity in vaccinated pigs as compared to control pigs is unexplained. Importantly, however, all vaccinates remained Anti-gpX ELISA positive PC.

Pigs from Trial 1 were not assayed to determine if any were latently infected with PRV. However, the peaks and troughs in Anti-gpX ELISA reactivity over time and the fact that the greatest group mean reactivity of both assays did not occur until 9 months PC is suggestive of latent infection with recrudescence.

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